SCIENTIFIC REPORT: ESF-Exchange Grant (Reference number 3620)

ESF activity: The Identification of Novel Genes and Biomarkers for Systemic Lupus Erythematosus

Project title: Production of RANKL by B cells in patients with SLE

Date of visit: 30/10/2011 to 05/05/2012

Applicant's Name: Banesa de Paz, Oviedo, Spain

Host´s name: Dr. Dagmar Scheel-Toellner and Professor Caroline Gordon. Birmingham, United Kingdom.

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Purpose of the visit

Systemic lupus erythematosus (SLE) is an autoimmune disease with heterogeneous clinical manifestations characterized by B lymphocyte hyper-reactivity and pathogenic auto-antibodies. The role of B cells, however, does not have to be limited to that of a precursor of antibody producing plasma cells, as they also have an important role as antigen presenting cells and producers of cytokines which are very relevant to the pathogenesis of lupus. There is extensive evidence for the activation and altered differentiation of B cells in patients with SLE.

The Rheumatology Research Group of University of Birmingham have developed a systematic approach to the *ex vivo* study of cytokine profiles in cell population. Among a range of interesting observations, the most intriguing were the expression of mRNA for a number of unexpected cytokines by B cells. RANKL expression by B cells was further confirmed by flow cytometry and by immunofluorescence staining of frozen sections of synovial tissue from patients with RA (Yeo L, Toellner KM, Salmon M, Filer A, Buckley CD, Raza K, Scheel-Toellner D. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. Ann Rheum Dis. 2011 Nov;70(11):2022-8. Epub 2011 Jul 8.) In ongoing work the Scheel-Toellner Lab is characterising the immunophenotype of the RANKL producing B cell population.

In this project we wanted to extend this work to the investigation of RANKL production by B cells circulating in patients with SLE and controls. We hypothesized that activated B cells in the peripheral blood of SLE patients produce RANKL. Previous studies in Birmingham and other institutes have shown an increased degree of osteoporosis and fractures in SLE patients *(C Yee, N Crabtree, J Skan, N Amft, S Bowman, D Situnayake, and C Gordon .Prevalence and predictors of fragility fractures in systemic lupus erythematosus. **Ann Rheum Dis**. 2005 January; 64(1): 111–113.)*. There is little information on RANKL expression in SLE patients in the published literature. RANKL production by activated B cells may therefore be part of a feedback loop affecting the generation of autoreactive B and T cells, and may have important roles beyond those associated with osteoporosis. Moreover, since the Scheel-Toellner Lab has now characterised the immunophenotype of the RANKL producing B cells in RA patients we used the same panel of morkers to look for the presence of related B cell populations in SLE patients, independently of their RANKL expression. Finally, we determined correlation with disease activity by the classic BILAG (British Isles Lupus Assessment Group) index ***classifying patients into active and inactive.***

Description of the work carried out during the visit

Peripheral blood samples from 40 SLE patients and 18 healthy controls were collected in heparinized tubes. PBMC were obtained from freshly-drawn blood by Ficoll-paque PLUS (GE Healthcare.Bio-Science AB) density gradient centrifugation. Patients fulfilling the American College of Rheumatology 1987 revised criteria for SLE were recruited for this pilot study and gave informed consent. Anti-dsDNA titer and disease activity (British Isles Lupus Assessment Group, BILAG) were determined at the time of sampling. A BILAG system score of B or more (≥5 points) was considered as a marker of active disease. Sex and age-matched healthy blood donors were recruited as controls. Signed informed consent was obtained from healthy blood donors and SLE patients prior to participation in the study, which was approved by the local research ethics committee.

Determination of RANKL expression in B cells population in SLE and healthy donors.

Specific population cells were gated by staining of specific markers. For characterization of B cells, blood cells were stained with anti-CD19 and anti-CD20. Highly extracellular expression of RANKL was analyzed by direct immunofluorescence staining using mouse anti RANKL-PE monoclonal antibody (eBioscience). All experiments were controlled using isotype- and concentration-matched irrelevant antibodies. Samples were read using a Cyan™ flow cytometer (Beckman Coulter, Fullerton, CA). Samples were subsequently analyzed using Summit software (Dako).

Determination of different markers of B cells in SLE and healthy donors.

B cells were stained by multiparameter flow cytometry with the following mABs: CD19-Pacific Blue (Biolegend), CD20-Pacific Orange (Invitrogen), CD27-APC (BD Pharmingen), CD38-PETR Invitrogen), CD138-FITC (eBioscience), IgM PerCP-Cy5.5 (Biolegend), IgD PE-Cy7 (Biolegend), CD11c APC-Cy7 (Biolegend), CD21 APC (Biolegend), CD11b FITC (Immunotools) , CD95 PerCP-Cy5.5 (Biolegend) and FcRL4 (Biolegend). The corresponding isotype and fluorochrome-matched controls were used to establish background levels. Single colour stainings were used to set compensate the flowcytometer settings for spectral overlap.
PBS with 0.5% bovine serum albumin was used as a diluent and washing buffer. FC analysis was performed using a Cyan™ flow cytometer (Beckman Coulter, Fullerton, CA).
Data were analysed using SUMMIT and FlowJo software. Results are expressed as the percentage of cells or as the MFI (Median fluorescence intensity) of the gated population.

Association of CD11c+ (CD19) with age and activity disease in SLE patients.

Determination of the membrane bound expression and intracellular BLyS by blood cell populations in SLE patients to complete the project title: *BLyS production by neutrophils in SLE*

Applicant's Name: Dr. Patricia Lopez. Oviedo, Spain

1. Description of the main results obtained

Expression of RANKL in SLE patients and healthy donors

Initially, we analyzed the surface levels of RANKL by isolated of PBMC from SLE patients and healthy donors by flow cytometry. We couldn't find significant differences in RANKL levels between patients and healthy donors (0.45 (2.47) *vs* 0.60 (2.22), p= 0.358). Similarly, the analysis of patients with active versus non-active disease did not show significant differences in levels of this marker (Figure 1). Therefore, we couldn´t report that B cells contribute to RANKL production like occurs in the inflamed rheumatoid joint recently published by the Rheumatology group of Birmingham University (Yeo L *et al*. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. **Ann Rheum Dis**. 2011 Nov;70(11):2022-8. Epub 2011 Jul 8.).

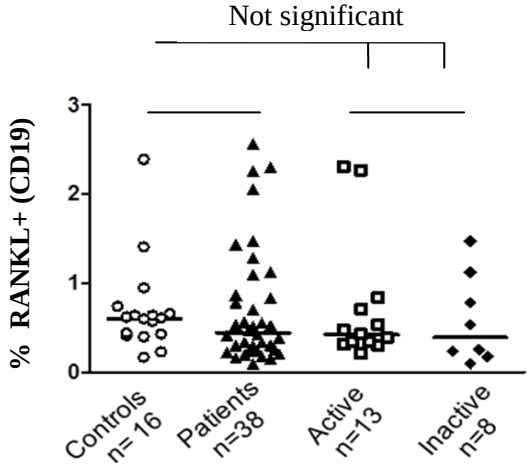

Figure 1. Percentage of RANKL+ cells among CD19 in Healthy donors and patients with SLE. Differences between different groups were assessed by the Mann-Whitney U-test.

CD11c+ B cell population is increased in SLE patients.

Since other work in the Scheel-Toellner lab has recently shown that the RANKL expressing B cell population in the synovial fluid of RA patients is a memory B cell subset largely $IgD^{-}/IgM^{-}/CD138^{-}/CD38^{low}/FCRL\text{-}4^{+}/CD27^{Int}/CD95^{high,}$ we used a combination of markers to investigate whether related B cells can be found in the peripheral blood of SLE patients. Also, a subset of CD11c + B cells has recently been described in the peripheral blood of RA patients over the age of 60. A similar age related subset of B cells has been described to be accumulated in ageing mice. These cells were termed ABC for age associated B cells. *(Rubtsow AV et al.* ***Blood*** *118 (5), 1305-1315, 2001; Hao Y et al.* ***Blood*** *118 (5), 1294-1304, 2011 ; Rakhmanov M et al.* ***Proc.Natl Acad. Sci. USA*** *106 (32), 13451-13456, 2009; Isnardi I et al.* ***Blood*** *115 (24), 5026-5036, 2010).*

We could observe increased numbers of $CD11c^{+}$ peripheral B cells in SLE patients compared with control group (3.73 *vs* 2.02, n=40 *vs* n=18, p= 0.0004). The frequency of these cells was not related to disease activity: SLE patients with active and inactive disease had significantly higher frequencies of CD11c- expressing B cells than control group (Figure 2). Further characterization of this new population in SLE patients revealed differences between CD11c+ and CD11c- B cells and found that these two subsets have very distinctive expression profiles for cell-surface proteins. The CD11c+ (CD19) population present high expression of CD11b , CD95, FcRL4, CD138, intermediate expression of CD27,and low expression of IgM, IgD, CD38 and CD21 when we compared with CD11c- B cells population (Figure 3). Thus, the cells in SLE patients displayed surface markers that were very similar to those that characterize ABCs in mice and contributes to complement the study of these population described for other authors. In summary, while we detected only a small population of RANKL expressing B cells in SLE patients, a related subset of B cells is significantly increased in the peripheral blood of these patients. Further work will need to be undertaken to investigate the functional role of CD11c+ B cells in SLE.

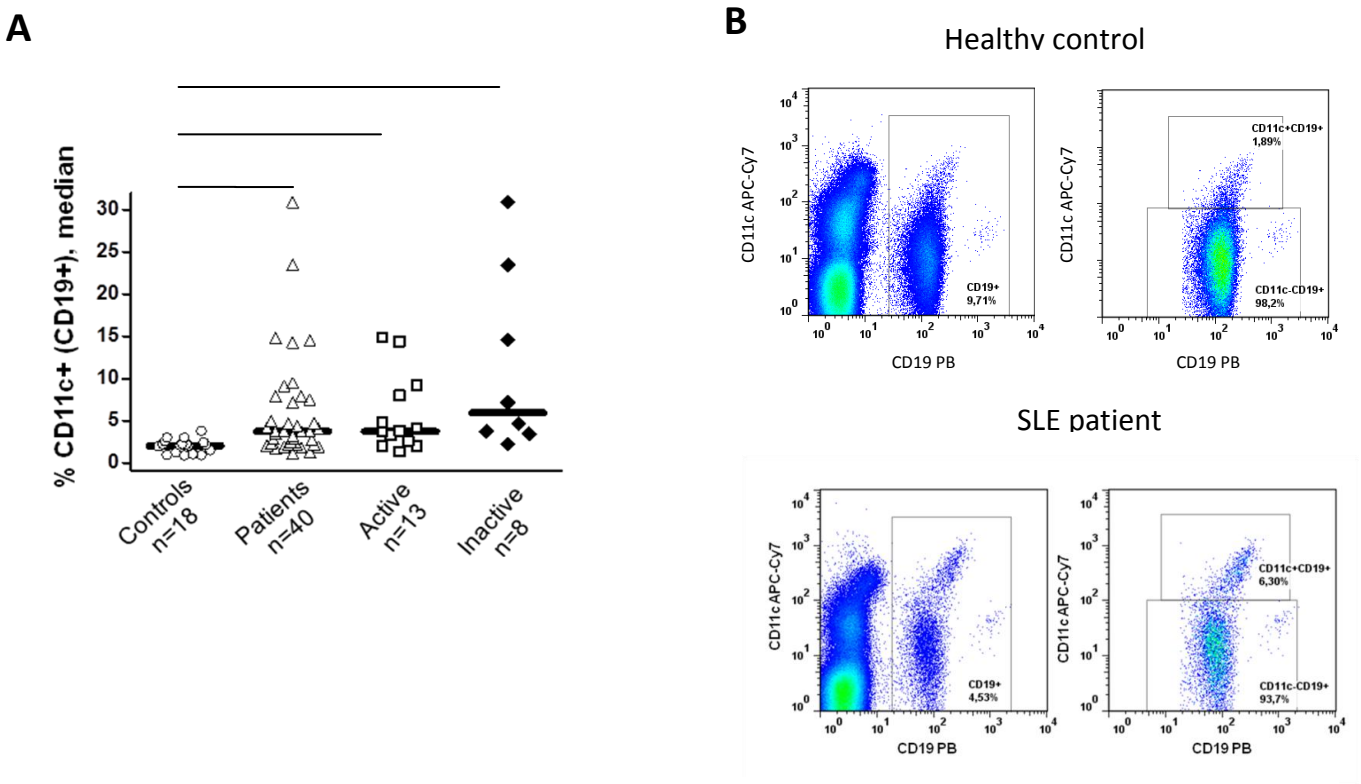

Figure 2. (A) Percentage of CD11c+ cells among CD19 in Healthy donors and patients with SLE. Differences between different groups were assessed by the Mann‐Whitney U-test. **(B)** CD11c expression in Bcells from SLE patients and Healthy donors. PBMCs from HCs and SLE patients were analysed by flow cytometry.

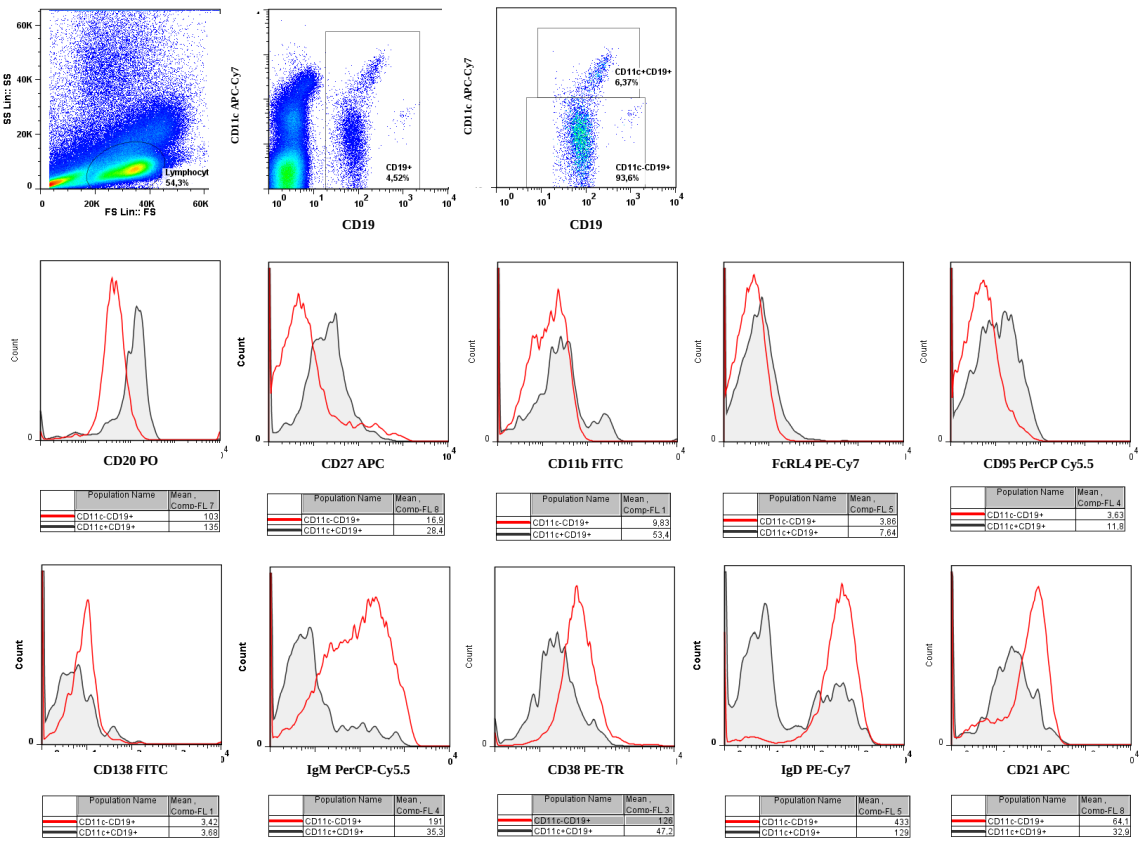

Figure 3. Flow cytometric characterization of CD11c+CD19+ (grey) and CD11c-CD19+ (red) in a representative SLE patient.

Presence of CD11c+CD19+ in peripheral blood of SLE patients correlated with age.

Other studies have described a population of B cells that seems similar to mouse ABCs (bearing CD11c and CD11b, but low levels of CD21). This population correlated with the age in female RA patients (). However, the authors detected only low levels of ABCs in the blood of SLE patients.The difference in our findings is probably due to the difference in the age of the patients studied.

Figure 4 shows a slight increase in the proportion of CD11c + CD19 cells in SLE patients while the frequency of this population remains stable in controls. Interestingly, we found a trend towards higher numbers of these cells in older patients but not in older healthy controls.

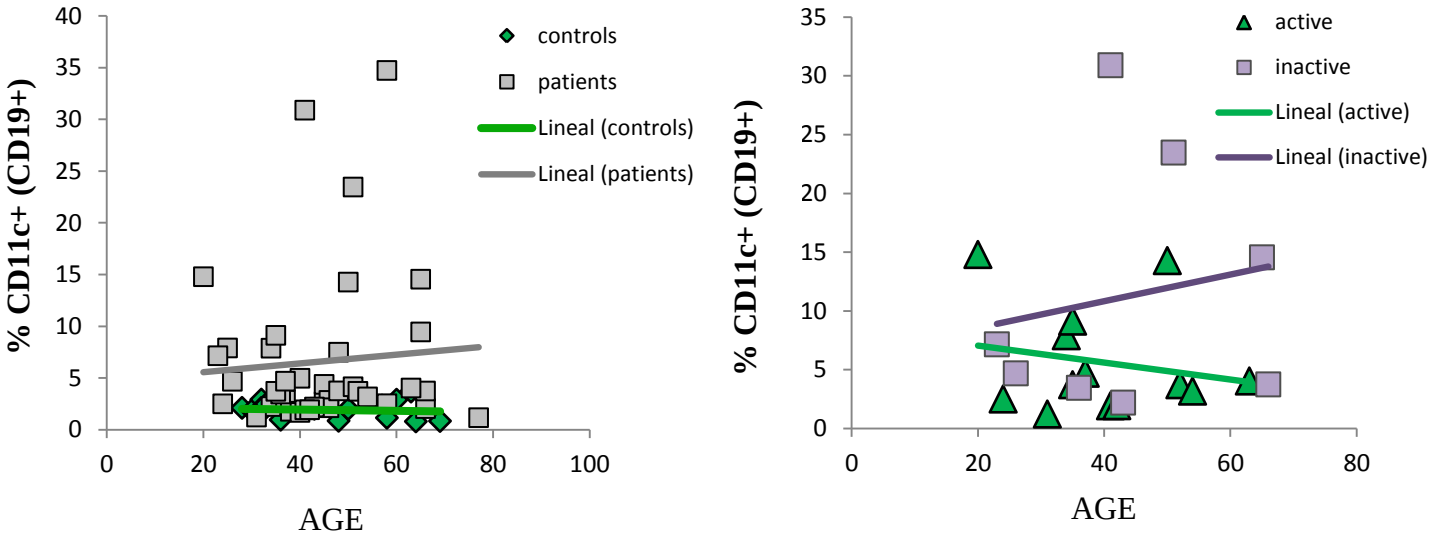

Figure 4. (A) Percentage of CD11c+ cells among CD19 in Healthy donors and patients with SLE and **(B)** active and inactive patients. The correlation between the percentage CD11c+CD19+ cells and age in SLE patients, active e inactive and healthy donors.

Statistical analysis

Values are shown throughout the paper as median and interquartile range (IR). The Mann-Whitney U test was used to evaluate differences between groups. The median intensity of fluorescence (**MIF**), which represents the density of expression of the surface marker was used to phenotype the CD11c B cell population.
All statistical analyses were performed using the SPSS software (version 15.0). Graphs were drawn using GraphPad Prism (version 4.0) or FlowJo software.

Future collaboration with host institution

In view of our results obtained from work performed in host institution of my Exchange Visit Grant, we will continue to collaborate in this project, so both Dr. Dagmar Scheel-Toellner and Prof. Caroline Gordon (Rheumatology Research Group, Birmingham University, United Kingdom) and Banesa de Paz (Autoimmunity Research Group managed by Dr. Ana Suarez, Oviedo University, Spain) will continue this work together.

Projected publications/articles resulting or to result from the grant

All results obtained in this project are being compiled now to be published in an international indexed scientific journal (***A CD11c+ B cell population in systemic lupus erythematosus patients****. De Paz B, Gordon C, Scheel-Toellner D*). Of course, as it has been indicated in the guidelines for the present Exchange Visit Travel Grant, ESF will be acknowledged in publications resulting from the grantee's work.